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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this study is to determine the role of a transcriptional factor, Msx2, in regulating initial induction of mammary anlagen and subsequent branching events that may require cooperative assistance by a few other molecules. Due to the time limitation of the funding period, we focused our attention on one key event that Msx2 gene may be an essential player, i.e. the initial inductive event to form the mammary gland. We took advantage the fact that we have Msx2 transgenic animals in that the Msx2 transgene is controlled by the Msx2 promoter. We have shown that this promoter is active in the mesenchyme of mammary anlagen around the time mammary gland is form in the mouse. We hypothesized that Msx2 transgenic animals should be able to rescue the mammary gland development in the Msx2 null mutants. As predicted, we were able to rescue mammary gland development in the Msx2 null mutants when the transgene is present. In addition, we also found that the transgene enhanced branching at the expense of extension. We also noticed that in a small percentage of Msx2 null mutants, mammary gland did development; however, mammary glands in these animals showed fewer branches. Taken together these results, we concluded that the Msx2 gene is essential for the initial induction event, and in addition it regulates numbers of branches.

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Introduction

Breast cancer is the second most common form of malignancy among women the second leading cause of fatality among women who have cancer. And yet, the underlying mechanisms that lead to the formation of mammary tumors remain unclear. Broadly, cancer can be defined as uncontrolled cell division that leads to starvation of normal cells and malfunction of normal physiological processes. Rapid cell division does occur during normal embryonic development and during tissue repair; however, these cell divisions are strictly controlled. Thus, understanding the molecular control mechanism of cell division and morphogenesis in normal development holds the key to unlock the mystery of cancer.

It's well established that ductal outgrowth in the mammary gland is controlled by ovarian hormones, especially, estrogen and progestrone. Results from studies of estrogen receptor null mutants and progestrone receptor null mutants have shown that ductal outgrowth is depended on stroma expressing estrogen receptor as well as progesterone receptor although branching requires progesterone receptors expressed by the epithelium. Experiments such as these firmly established the importance of epithelial-stromal interactions in regulating developmental events in the mammary gland. However, the molecular mechanisms by which these processes function are still poorly understood. And yet a clear understanding of the fundamental molecular mechanism that regulates the normal developmental processes holds the key to the creation of innovative methods for early diagnosis and treatment of breast cancer.

One group of molecules that have been shown to have critical function in the development of epidermal appendages including mammary gland are the Msx homeodomain-containing proteins, the *Msx1* and *Msx2*. The *Msx* gene family is named for the Drosophila muscle segment homeobox (*msh*) gene, which is expressed in specific mesodermal and neuronal cell populations during embryogenesis (Walldorf *et al.*, 1989; D'Alessio and Frasch, 1996; Isshiki et al, 1997). Loss-of-function mutation of *msh* led to alterations of neuroblast cell fate, such that the dorsal cells took on ventral neuronal cell fates (Isshiki *et al.*, 1997). Targeted overexpression of the *msh* gene resulted in severe disruption of the proper development of the midline and ventral neuroblasts (Isshiki *et al.*, 1997). Genetic analyses of two other *msh*-related genes, *tinman* and *bagpipe*, have shown that both genes are required for the determination of cell fates in subpopulations of the dorsal mesoderm in Drosophila embryos (Bodmer, 1993; Azpiazu and Frasch, 1993).

Homology screening approaches have been used to isolate *msh*-related, or *Msx* genes, from a variety of non-vertebrate and vertebrate organisms, including Hydra (phylum Coelenterata) (Davidson, 1995), sea urchin (phylum Echinodermata) (Dobias *et al.*, 1997), ascidian (phylum Urochordata) (Holland, 1991; Ma *et al.*, 1996) Amphioxus (phylum Chordata) (Holland *et al.*, 1994), and representatives of all vertebrates classes (Holland, 1991; Bell, *et al.*, 1993; Davidson *et al.*, 1995). Mammals are known to possess three *Msx* genes, designated *Msx1*, *2* and *3* (Davidson, 1995). General features of the *Msx* genes are (i) a non-clustered organization; (ii) a distinct homeodomain sequence, substantially different from sequences of the clustered *Hox* genes; (iii) a striking conservation of homeodomain amino acid sequences across wide phylogenetic distances: only one amino acid, for example, distinguishes the 70 amino acid homeodomain regions

of the sea urchin *SpMsx* gene and the mouse *Msx2* gene--in spite of more than 600 million years of separate evolution (Bell *et al.*, 1993).

Such a widespread phylogenetic distribution and such extreme conservation of amino acid sequence suggest that the *msh* gene family performs fundamental and conserved tasks throughout the metazoa. Consistent with the view is the striking similarity in the patterns of expression of *Msx* genes in early embryos of highly divergent organisms. For example, in Xenopus, *Msx* transcripts are found in dorsal mesoderm after its involution over the dorsal lip, and subsequently in the overlying neural ectoderm, including the cranial neural crest (Su *et al.*, 1991). Strikingly, elements of this pattern are recognizable even in mammalian embryos. We and others have identified *Msx1* and *Msx2* transcripts in the limb mesenchyme and in the overlying apical ectodermal ridge, in the tooth bud, in hair follicles, and in cranial neural crest and its derivatives (Davidson *et al.*, 1991; Coelho *et al.*, 1991a, b; Robert *et al.*, 1991; MacKenzie *et al.*, 1992; Satokata and Maas, 1994; Chen *et al.*, 1996; Davidson, 1995). A common feature of these sites is that they are tissues actively engaged in inductive interactions. In the skull, *Msx1* and *Msx2* transcripts are localized in the ectomesenchyme of the developing skull and sutures (Mackenzie *et al.*, 1991a, b; Liu *et al.*, 1999) where interactions between the suture and the underlying dura determine the growth of the flat bones of the skull (Opperman *et al.*, 1993; Opperman et al.1995). These genes act as general regulators of proliferation/ differentiation and active participants of apoptosis (Graham *et al.*, 1994; Odelberg *et al.*, 2001; Holme *et al.*, 2000).

Msx2 gain of function phenotypes in humans suggest that *Msx2* gene is an essential player in calvarial morphogenesis. Jabs et al (1993) demonstrated that the human *Msx2* gene is mutated in individuals affected with Boston type craniosynostosis, an autosomal dominant disorder of cranial patterning characterized by the premature fusion of calvarial bones and consequent abnormal skull shape. We have introduced the Boston mutation--a Pro-148->His substitution in the N-terminal arm (position 7) of the homeodomain--into the mouse *Msx2* gene. We have shown that transgenic mice bearing this mutant gene under the control of the *Msx2* promoter or a heterologous promoter (CMV or TIMP-1) exhibit premature fusion of calvarial bones, and thus recreate the major feature of the human defect (Liu *et al.*, 1995). We demonstrated further that overexpression of the wild type *Msx2* gene elicits a similar phenotype--a result that suggests that the Pro 148-> His mutation acts by a dominant positive mechanism (Liu *et al.*, 1999).

Early in development, *Msx2* transcripts were detected in the invaginating mammary ectoderm among other sites (Philippard *et al.*, 1996; Friedmann and Daniel, 1996). Shortly after ectodermal invagination, it's expression is localized to the mesenchyme surrounding the ectodermal bud. In postnatal mammary gland, *Msx2* expression is detected in ductal mesenchyme and declines sharpely early in pregnancy and then reinduced during the involution phase. Intriguingly, *Msx2* expression can be modulated by estrogen and is myoepithelial dependent (Friedmann and Daniel, 1996). In the *Msx2* null mutants, both ductal elongation and branching is severely affected in prepubertal mammary glands and in 30% of *Msx2* knockout animals, mammary gland arrested at the bud stage (1) which is a phenocopy of PTH/PTHrP receptor knockout (Wysolmerski te al., 1998). And interestingly, in the *Msx2* knockout animals, the expression of PTH/PTHrP receptor is reduced in long bones and trabecular bone volume is reduced, a similar defect observed in PTH/PTHrP heterozygous knockout animals.

These intriguing observations prompt me to formulate following hypotheses: that modulation of *Msx2* expression by estrogen is a direct regulation of gene expression through estrogen receptor binding to the *Msx2* promoter; and that *Msx2* regulates ductal branching by modulating PTH/PTHrP receptor expression in the ductual mesenchyme. Our specific aims are: (1) to investigate the molecular mechanism of estrogen/estrogen receptor regulation of *Msx2* transcription; (2) to examine the molecular interactions between the *Msx2* gene and PTH/PTHrP signaling pathway in regulating ductual branching.

Results

To test the first hypothesis, in collaboration with Dr. Kenneth Korach at NIH we have performed northern blot hybridization of RNAs obtained from the estrogen receptor knockout animals using *Msx2* cDNA probe. The blot showed that *Msx2* expression was reduced by less than two folds in the estrogen receptor knockout animals although *Msx2* transcripts were expressed in detectable levels. This result showed that the expression of *Msx2* does not require functional estrogen receptor. The mammary gland phenotype in the ER null animals may not entirely due to reduction in *Msx2* gene expression, suggesting that a complex genetic interaction appears to dictate early stages of mammary gland development and may require additional genetic components.

In our preliminary studies prior to the submission of this grant proposal, we have shown that a 400bp fragment of the *Msx2* promoter targets reporter expression to the stroma of the developing mammary gland in the mouse embryo (Figure 1). To extend this result further, we examined the activity of this DNA fragment in the mammary glands of postnatal animals. To our surprise, this promoter fragment is silent in postnatal mammary glands. 17beta-estradiol pellet implantations on to mammary fat pads also failed to induce reporter gene expression in transgenic animals. These results suggested that this 400bp fragment of the *Msx2* promoter constitutes regulatory elements that restrict its transcriptional activity in the mammary gland during early stages of mammary gland development and it does not respond to stimulation by estrogen. Since this DNA fragment is active only in stroma cells of the embryonic mammary gland, transfection assay to examine its transcriptional activity in epithelium-derived MCF7 cell line yielded negative results. Nevertheless, this 400bp DNA regulatory sequence in the *Msx2* promoter is the first promoter studied that targets gene expression to the mammary stroma of the embryonic mammary analgen. More studies are needed to identify regulatory factors that control its activity during initial stages of mammary gland development.

We have examined mammary gland phenotypes in both the *Msx2* transgenic animals and the *Msx2* null animals. The *Msx2* transgene was under the control of a 5.2kb *Msx2* promoter (Liu *et al.*, 1994, Liu *et al.*, 1999). In the transgenic animals, more lateral branches were observed (Figure 2B) although the gland did not extend as far as that of the nontrangenic mammary gland (Figure 2A). In contrast, the development of mammary glands in *Msx2* null mutant animals were either retarded or inhibited (Figure 2C and 2D). Mammary ducts were dilated with large terminal end buds (TEBs) (Figure 2C). These results suggest that *Msx2* gene expression is required for mammary gland development. Too much *Msx2* expression however promotes lateral branching of the mammary gland at

the expense of ductal extension. Based on the phenotype of the *Msx2* transgenic animal, we hypothesize that the *Msx2* gene participates in the development of the mammary gland by exerting its regulatory function in (i) the initial phase of mammary bud induction and in (ii) determining branching points.

Interestingly, several other genes have been shown to participate in the regulatory decisions of side-branching. Among these are progesterone and progesterone receptor, prolactin and prolactin receptor, C/EBPβ (Lydon *et al.*, 1995; Shyamala *et al.*, 1998; Atwood *et al.*, 2000; Ormandy *et al.*, 1997; Robinson *et al.*, 1998; Seagroves *et al.*, 1998; Robison *et al.*, 2000). It will be important to examine genetic interactions among molecules in order to decipher the regulatory hiearchies that dictate normal mammary gland development.

Our plan of action in the remaining funding period is to perform an extensive analysis of the *Msx2* overexpression transgenic and rescued phenotypes.

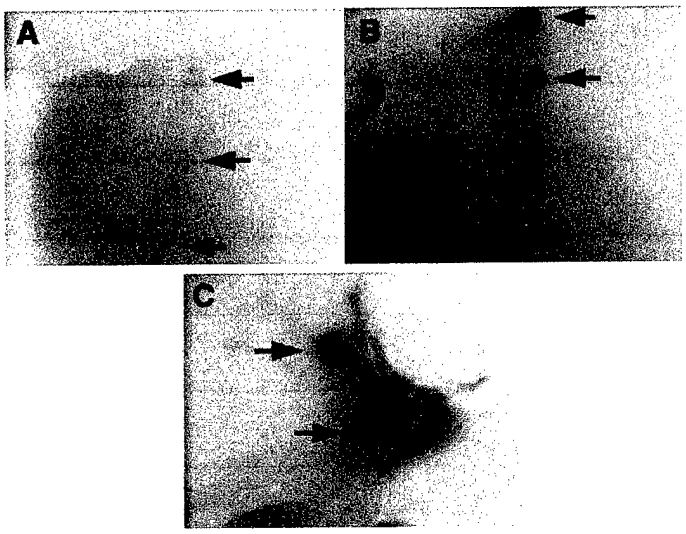

Figure 1: A 400bp 5' up-steam DNA fragment in the *Msx2* promoter targets the lacZ reporter expression in the mesenchyme surrounding the mammary epithelial bud. (A) In an E12 embryo, the expression of the lacZ reporter in the mammary gland anlagens was significantly reduced in the *Msx2* null background (arrows). (B) The expression of the lacZ reporter in the mammary anlagen was intense in a littermate control. (C) In an E14 embryo, lacZ expression level appeared to be higher.

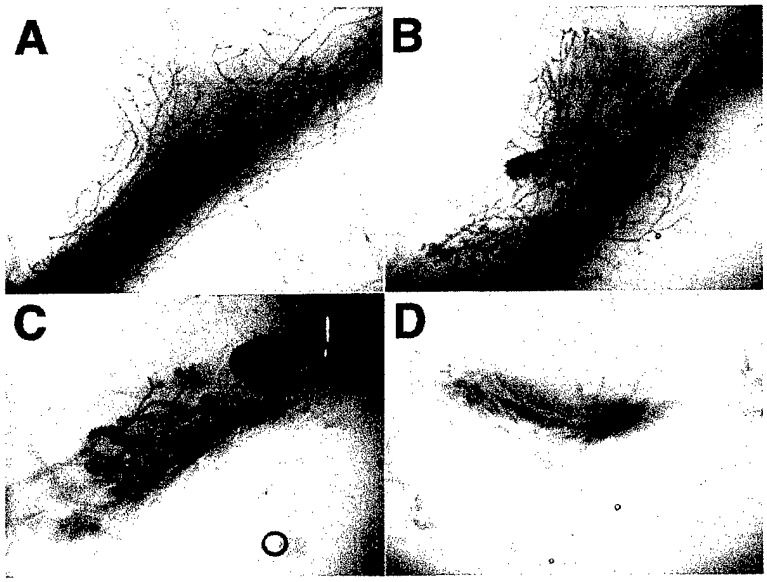

Figure 2: Whole-mount analysis of mammary tissue from 8 week-old virgin animals. (A) Mammary tissue from a wildtype virgin showed extensive ductal elongation. Some side branching was observed. (B) Mammary tissue from a *Msx2* transgenic animal displayed elaborate amount of side branches. Ductal elongation was retarded in comparison to the wildtype control in A. (C) and (D) Mammary tissues from two different *Msx2* null mutants showed that both ductal elongation and branching were severely inhibited.

Key Research Accomplishments

1. *Msx2* gene activity is required for induction and branching of mouse mammary gland.

2. Overexpression of *Msx2* gene in the mammary tissue induces more side-branching.

3. The 400bp *Msx2* regulatory sequences do not respond to estrogen induction *in vivo*.

4. *Msx2* gene expression in Estrogen Receptor α (ERα) null mutant mammary tissue was not significantly affected (<2 fold), indicating that the *Msx2* gene is not required for ERα function.

Reportable Outcomes

Not at this time.

Conclusion

Based on phenotypes of the *Msx2* null and transgenic animals, we can conclude that the *Msx2* gene is essential for normal mouse mammary gland development. *Msx2* gene activity is required for induction and branching of mouse mammary gland. Over-expression of the *Msx2* gene in the mammary tissue induces more side-branching, suggesting that *Msx2* gene may be required for determining the branching points on the TEB. Our study of the 400bp *Msx2* regulatory sequences fail to show induction by estrogen and *Msx2* gene expression in Estrogen Receptor α (ERα) null mutant mammary tissue was not significantly affected (<2 fold), indicating that the *Msx2* gene is not required for ERα function.

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